

**Amendments to the Claims:**

This listing of claims will replace all prior versions, and listings, of claims in the application:

**Listing of Claims:**

- 1-15. (cancelled)
16. (withdrawn) A recombinant nucleic acid molecule comprising a sequence encoding the modified pneumolysin polypeptides of any one of claims 1 to 15.
17. (withdrawn) A recombinant nucleic acid molecule comprising the following pneumolysin nucleic acid sequence:

ATGGCAAATA	AAGCAGTAAA	TGACTTTATA	CTAGCTATGA	40
ATTACGATAA	AAAGAAACTC	TTGACCCATC	AGGGAGAAAG	80
TATTGAAAAT	CGTTTCATCA	AAGAGGGTAA	TCAGCTACCC	120
GATGAGTTTG	TTGTTATCGA	AAGAAAGAAG	CGGAGCTTGT	160
CGACAAATAC	AAGTGATATT	TCTGTAACAG	CTACCAACGA	200
CAGTCGCCTC	TATCCTGGAG	CACTTCTCGT	AGTGGATGAG	240
ACCTTGTAG	AGAATAATCC	CACTCTTCTT	GCGGTCGATC	280
GTGCTCCGAT	GACTTATAGT	ATTGATTTCG	CTGGTTTGGC	320
AAGTAGCGAT	AGCTTTCTCC	AAGTGGAAAGA	TCCCAGCAAT	360
TCAAGTGTTC	GCGGAGCGGT	AAACGATTTG	TTGGCTAAGT	400
GGCATCAAGA	TTATGGTCAG	GTCAATAATG	TCCCAGCTAG	440
AATGCAGTAT	GAAAAAAATCA	CGGCTCACAG	CATGGAACAA	480
CTCAAGGTCA	AGTTGGTTC	TGACTTTGAA	AAGACAGGGA	520
ATTCTCTTGA	TATTGATTTT	AACTCTGTCC	ATTCAAGGCGA	560
AAAGCAGATT	CAGATTGTTA	ATTTTAAGCA	GATTTATTAT	600
ACAGTCAGCG	TAGACGCTGT	TAAAAAATCCA	GGAGATGTGT	640
TTCAAGATAAC	TGTAACGGTA	GAGGATTAA	AACAGAGAGG	680
AATTCTGCA	GAGCGTCCTT	TGGTCTATAT	TTCGAGTGT	720
GCTTATGGGC	GCCAAGTCTA	TCTCAAGTTG	GAAACCACGA	760
GTAAGAGTGA	TGAAGTAGAG	GCTGCTTTG	AAGCTTGAT	800
AAAAGGAGTC	AAGGTAGCTC	CTCAGACAGA	GTGGAAGCAG	840
ATTTTGGACA	ATACAGAAAGT	GAAGGCGGTT	ATTTAGGGG	880
GCGACCCAAG	TTCGGGTGCC	CGAGTTGAA	CAGGCAAGGT	920
GGATATGGTA	GAGGACTTGA	TTCAAGAAGG	CAGTCGCTTT	960
ACAGCAGATC	ATCCAGGCTT	GCCGATTCC	TATACAACCT	1000
CTTTTTTACG	TGACAATGTA	GTTGCGACCT	TTCAAAATAG	1040
TACAGACTAT	GTTGAGACTA	AGGTTACAGC	TTACAGAAC	1080
GGAGATTAC	TGCTGGATCA	TAGTGGTGCC	TATGTTGCC	1120
AATATTATAT	TACTTGGAAAT	GAATTATCCT	ATGATCATCA	1160

AGGTAAGGAA	GTCTTGACTC	CTAAGGCTTG	GGACAGAAAT	1200
GGGCAGGATT	TAACGGCTCA	CTTTACCCT	AGTATTCCCT	1240
TAAAAGGGAA	TGTTCGTAAT	CTCTCTGTCA	AAATTAGAGA	1280
GTGTACCGGG	CTTGCTTGGG	AATGGTGGCG	TACGGTTTAT	1320
GAAAAAAACCG	ATTGCCACT	AGTGCCTAAG	CGGACGATT	1360
CTATTGGGG	AACAACCTCTC	TATCCGCAGG	TAGAAGATAA	1400
GGTAGAAAAT	GAC	<u>(SEQ ID NO: 1)</u>		1413

and wherein said nucleic acid sequence comprises one or more of the nucleotide substitutions selected from the group consisting of:

A-50G, G-54T, T-181C, A-196T and T-302C;

or

A-122G, A-514G, T-583A and A-764G;

or

A-187T, T-380A, A-382C and T-443A;

or

T-98C, T-137C, T-248C, T-717A and A-770G;

or

T-134C, A-305G, A-566G and T-583G;

T-583G;

T-583A;

T-443A;

and

T-181C.

18. (withdrawn) The recombinant nucleic acid molecule of claim 16 as contained in a vector such as a plasmid, cosmid, bacteriophage or yeast artificial chromosome.
19. (withdrawn) A microorganism comprising the nucleic acid molecule of claim 16.
20. (withdrawn) The microorganism according to claim 19, wherein the microorganism is selected from the group consisting of bacteria, yeast, mammalian or insect cells.
21. (withdrawn) The microorganism according to claim 20, wherein the microorganism is *E. coli*.

22-26. (cancelled)

27. (withdrawn) A method for killing bacteria comprising contacting said bacteria with antibodies to an immunogenic molecule comprising the modified pneumolysin according to claim 1 in the presence of complement.

28. (withdrawn) The method according to claim 27, wherein the immunogenic molecule is a polysaccharide-polypeptide conjugate where in the polysaccharide is a bacterial capsular polysaccharide.

29. (withdrawn) A method for immunization of mammals comprising administrating the vaccine of claim 24 to said mammals.

30. (withdrawn) A method for obtaining modified pneumolysin polypeptides having reduced hemolytic activity and being suitable for eliciting an immunogenetic response which is cross-reactive with wild-type pneumolysin comprising the steps of:

- a) randomly mutating a nucleic acid molecule encoding for wild-type pneumolysin to produce mutated nucleic acid molecules encoding modified pneumolysin polypeptides and expressing the mutated nucleic acid molecules in host cells;
- b) assaying the modified polypeptide expressed by the host cells for hemolytic activity;
- c) identifying the modified pneumolysins polypeptides having substantially similar molecular weight as native wild-type pneumolysin and which are refoldable.

31-34. (cancelled)

35. (currently amended) A modified pneumolysin polypeptide, wherein the modification of the polypeptide comprises substituting at least one amino acid of SEQ ID NO:3, and wherein said substitution is at a position selected from the group consisting of positions 61, 148, and 195, and more than one amino acid substitution selected from the group consisting of positions 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, and wherein when said modified pneumolysin polypeptide possesses only one substitution, said substitution is selected from the group

consisting of positions 61, 148, and 195, and wherein said modified pneumolysin polypeptide having ~~said~~ <sup>one or more than one</sup> amino acid substitution is soluble, elicits antibodies which are cross-reactive with wild-type pneumolysin, and has attenuated hemolytic activity.

36. (previously added) The polypeptide according to claim 35, wherein the hemolytic activity is less than 25% compared to wild-type pneumolysin.

37. (previously added) The polypeptide according to claim 36, wherein the hemolytic activity is less than 1% compared to wild-type pneumolysin.

38-41. (cancelled)

42. (previously added) The polypeptide according to claim 35, wherein the polypeptide is selected from the group consisting of pNVJ1, pNVJ20, pNVJ22, pNVJ45, pNVJ56, pNV103, pNV207, pNV111, and pNV211.

43. (previously added) Modified pneumolysin polypeptide pNVJ1.

44. (previously added) Modified pneumolysin polypeptide pNVJ20.

45. (previously added) Modified pneumolysin polypeptide pNVJ22.

46. (previously added) Modified pneumolysin polypeptide pNVJ45.

47. (previously added) Modified pneumolysin polypeptide pNVJ56.

48. (previously added) Modified pneumolysin polypeptide pNV103.

49. (previously added) Modified pneumolysin polypeptide pNV207.

50. (previously added) Modified pneumolysin polypeptide pNV111.

51. (previously added) Modified pneumolysin polypeptide pNV211.

52. (cancelled)

53. (currently amended) A The modified pneumolysin polypeptide according to claim 35, wherein the polypeptide is soluble, elicits antibodies which are cross-reactive with wild-type pneumolysin, has attenuated hemolytic activity, and wherein the modification of the polypeptide is obtained by randomly mutating a nucleic acid molecule encoding a pneumolysin polypeptide, ~~and wherein the modified pneumolysin polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.~~

54-60. (cancelled)

61. (currently amended) The conjugate modified pneumolysin polypeptide conjugated to a polysaccharide according to claim 5360, wherein the polysaccharide is derived from a bacterium selected from the group consisting of *Haemophilus influenzae* type b; meningococcus group A, B or C; group B streptococcus type Ia, Ib, II, III, V or VIII; and one or more of serotypes 1-23 of *S. pneumoniae*.

62. (currently amended) A vaccine comprising the modified pneumolysin polypeptide according to claim 35 and a pharmaceutically acceptable carrier.

63. (previously added) The vaccine according to claim 62, wherein the polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.

64. (previously amended) The vaccine according to claim 63, wherein the bacterial polysaccharide is from a bacterium selected from the group consisting of *Haemophilus influenzae* type b; meningococcus group A, B, or C; group A streptococcus or group B streptococcus type Ia, Ib, II, III, V, or VIII; and one or more of serotypes 1-23 of *S. pneumoniae*.

65. (currently amended) The modified pneumolysin polypeptide according to claim 35, wherein ~~said modified pneumolysin polypeptide having only one amino acid substitution at position 61~~ is a proline or hydroxyproline substitution ~~at position 61~~.

66. (currently amended) The modified pneumolysin polypeptide according to claim 35, wherein ~~said modified pneumolysin polypeptide having only one amino acid substitution at position 148~~ is a lysine, arginine or histidine substitution ~~at position 148~~.

67. (currently amended) The modified pneumolysin polypeptide according to claim 35, wherein ~~said modified pneumolysin polypeptide having only one amino acid substitution at position 195~~ is a leucine, glycine, alanine, valine or isoleucine substitution ~~at position 195~~.

68. (currently amended) The modified pneumolysin polypeptide according to claim 35, wherein ~~said modified pneumolysin polypeptide having only one amino acid~~

substitution at position 243 is an arginine, valine, glutamic acid, or serine substitution at position 243.

69. (currently amended) The modified pneumolysin polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at positions 17, 18, 61, 66 and 101.

70. (currently amended) The modified pneumolysin polypeptide according to claim 69, wherein the substitutions consist of arginine at position 17, asparagine at position 18, proline at position 61, tyrosine at position 66, and threonine at position 101.

71. (currently amended) The modified pneumolysin polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at positions 41, 172, 195 and 255.

72. (currently amended) The modified pneumolysin polypeptide according to claim 71, wherein the substitutions consist of glycine at position 41, alanine at position 172, isoleucine at position 195, and glycine at position 255.

73. (currently amended) The modified pneumolysin polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at positions 63, 127, 128 and 148.

74. (currently amended) The modified pneumolysin polypeptide according to claim 73, wherein the substitutions consist of serine at position 63, glutamic acid at position 127, histidine at position 128, and lysine at position 148.

75. (currently amended) The modified pneumolysin polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at positions 33, 46, 83, 239 and 257.

76. (currently amended) The modified pneumolysin polypeptide according to claim 75, wherein the substitutions consist of threonine at position 33, threonine at position 46, serine at position 83, arginine at position 239 and glycine at position 257.

77. (currently amended) The modified pneumolysin polypeptide according to claim 75, wherein the substitutions at positions 33, 46 and 83 are either a serine, threonine, asparagine, glutamine, tyrosine or cysteine; the substitutions at position 239 is either a

lysine, arginine or histidine; and the substitution at position 257 is either a leucine, glycine, alanine, isoleucine or valine.

78. (currently amended) The modified pneumolysin polypeptide according to claim 35, wherein the modification of the polypeptide comprises a combination of substitutions at positions 45, 102, 189 and 195.

79. (currently amended) The modified pneumolysin polypeptide according to claim 78, wherein the substitutions consist of alanine at position 45, glycine at position 102, arginine at position 189, and valine at position 195.

80. (currently amended) A modified pneumolysin polypeptide, wherein the modification of the polypeptide comprises substituting at least one amino acid sequence having of SEQ ID NO:3, and wherein said substitution is at a position selected from the group consisting of positions 61, 148, and 195, and more than one amino acid substitutions selected from the group consisting of positions 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, and wherein when said modified pneumolysin polypeptide possesses only one substitution, said substitution is selected from the group consisting of positions 61, 148, and 195, and wherein said modified pneumolysin polypeptide having one or more amino acid substitutions is soluble, elicits antibodies which are cross-reactive with wild-type pneumolysin, and has attenuated hemolytic activity, wherein the amino acid substitution at position 17 is arginine; wherein the amino acid substitution at position 18 is asparagine; wherein the amino acid substitution at positions 33, 46, and 83 is selected from the group consisting of serine, threonine, asparagine, glutamine, tyrosine, and cysteine; wherein the amino acid substitution at position 41 is glycine; wherein the amino acid substitution at position 45 is alanine; wherein the amino acid substitution at position 61 is a proline or hydroxyproline; wherein the amino acid substitution at position 63 is serine; wherein the amino acid substitution at position 66 is tyrosine; wherein the amino acid substitution at position 101 is threonine; wherein the amino acid substitution at position 102 is glycine; wherein the amino acid substitution at position 127 is glutamic acid; wherein the amino acid substitution at position 148 is selected from the group consisting of lysine, arginine

and histidine; wherein the amino acid substitution at position 172 is alanine; wherein the amino acid substitution at position 189 is arginine; wherein the amino acid substitution at position 195 is selected from the group consisting of leucine, glycine, alanine, valine and isoleucine; wherein the amino acid substitution at position 239 is selected from the group consisting of lysine, arginine, and histidine; wherein the amino acid substitution at position 243 is selected from the group consisting of arginine, valine, glutamic acid, and serine; wherein the amino acid substitution at position 255 is glycine; and wherein the amino acid substitution at position 257 is selected from the group consisting of leucine, glycine, alanine, isoleucine, and valine.